Appendix A

Diversity amongst anti-hapten antibodies

This appendix outlines some of the studies that have been performed to determine the extent of variation between different antibody molecules raised against a common, simple determinant. Four different examples are provided, in which four different haptens were used as the immunogen. Copies of the respective academic publications are provided herewith.

These and other studies demonstrate that widely diverse sequences are generated during the immune response to any particular antigen, due to a combination of V, D, and J region selection, VJ and VDJ splicing, and somatic mutation.

The operation of these events make it essentially impossible to identically reproduce an antibody with a somatically mutated sequence by immunizing a second animal. Clones producing antibody molecules with identical sequences have been described only: a) when based on a nearly *un-mutated germ-line sequence*, in which case the antibody is observable during the primary response; or b) when obtained from the *same* animal, in which case the responsible antibody-producing cells are derived from the same clonal progenitor.

Example 1:

Nahmias et al. (1988, J. Immunol. 140:1304) raised a panel of 14 monoclonal antibodies against the ß-adrenergic hapten alprenolol, also using the same mouse strain. The 14 antibodies utilized at least seven V_L, four J_L, eight V_H, and three J_H genes (Table I), and also demonstrated extensive splicing and mutational diversity (Figures 3-5). Only three pairs of hybridomas used the same H and L chain gene rearrangements; each related pair was obtained from a single mouse and was apparently derived from the same clone (page 1308, ¶ 3).

Exampl 2:

Stenzel-Poore et al. (1989, J. Immunol. 143:4123) raised a panel of monoclonal antibodies against phosphocholine (PC) in BALB/c mice and F1 hybrids. Fourteen monoclonal antibodies were selected from the high affinity Group II anti-PC that emerge in the secondary response. The 14 antibodies utilized four V_L, six J_L, six to eleven V_H, four J_H genes, and more than five D genes (Table III). The antibodies each had an average of 3.6 replacement mutations in the heavy chain and 3.1 replacement mutations in the light chain (Table V). Here, as in other studies, the mutations were found *throughout the variable region* – they were enriched in the complementarity determining region (CDR), but also occurred in the framework (Table V).

Example 3:

Blier et al. (1987, J. Immunol. 139:3996) obtained monoclonal antibodies specific for 4-hydroxy-3-nitrophenyl acetate (NP). Twenty eight hybridomas were obtained during the secondary response in the *same mouse*. Fourteen were derived from different clones. Amongst the 14 families, about 3 different V_H, 3 different D and 3 different J_H were used (Table I). Nine families used the same V_H and D region genes, but were all spliced differently to create differences in the CDR4 region (Figure 1). Amongst the 28 antibody panel, there was an average of *8.1 amino acid replacements in each heavy chain variable region* (Table III). On average, *2.5 replacements had occurred after divergence of members of each clone family* (Table III). This indicates that somatic mutation is an *ongoing* process within B-cell clones during the immune response.

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Exampl 4:

Leahy et al. (1988, Proc. Natl. Acad. Sci. USA 85:3661) raised a panel of 12 monoclonal antibodies against a DNP spin-label hapten. The mouse strain used was BALB/c, the same as was used in the disclosure of the instant application to develop 1A7. The amino acid sequences of both the heavy and light chains of the Leahy panel demonstrate that different clones are derived from different germ-line V genes, exhibit junctional diversity around the splice sites, and show mutational divergence from common germ-line precursor sequences. As a result, the sequences are dramatically different amongst the antibodies.

These sequences are reproduced on the next page.

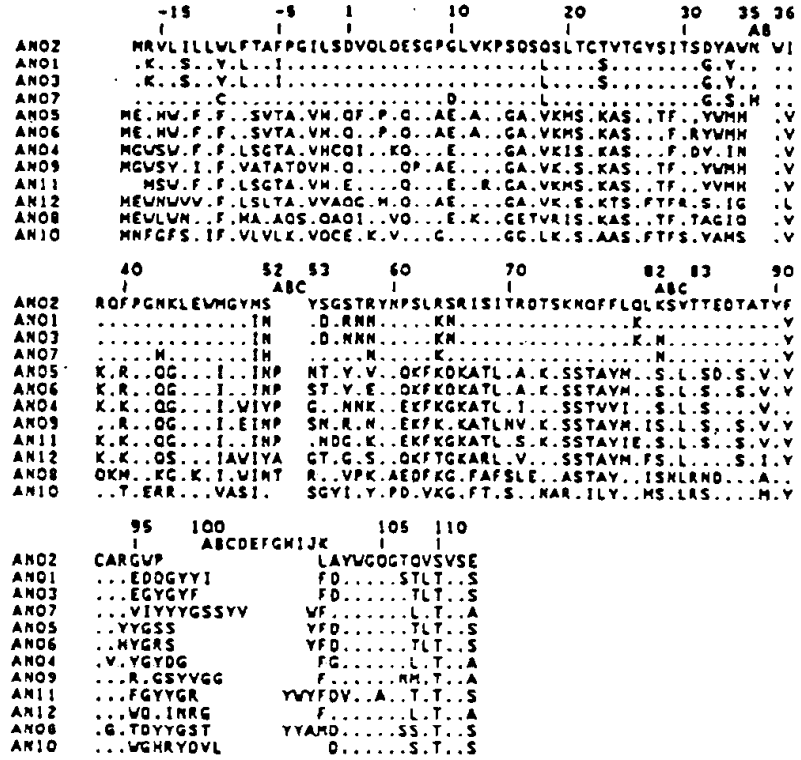

FIG. 1. Deduced amino acid sequences of the *V* regions of the heavy chains of the anti-DNP-SL monoclonal antibodies AN01–AN12.

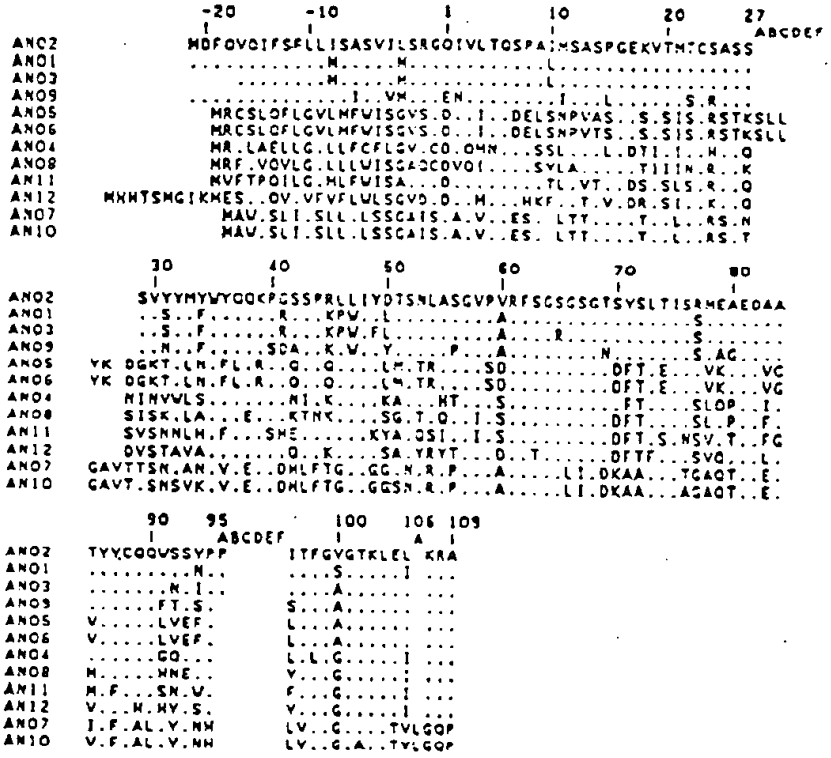

FIG. 2. Deduced amino acid sequences of the *V* regions of the light chains of the anti-DNP-SL monoclonal antibodies AN01–AN12.

Appendix B

Probability of reproducing 11D10 in a second hybridoma from a different animal

Background:

[illegible]	• Multiple VJ gene choices and splices are allowed to form suitable prototype light chains to bind any particular antigen. • Multiple VDJ gene choices and splices are allowed to form suitable prototype heavy chains to bind any particular antigen. • At least ~83% of the amino acids can vary from prototype sequences. • At least ~4 variations are allowed on average in the other positions. (The 12 sequences obtained by Leahy et al. have an average of ~3.5 variations in the 83% of the positions that vary. 12 is a small sample size for such a variable systems, and these numbers are therefore underestimates. On average, any codon can mutate to codons for 6 distinct amino acids with a single base change.) • A plurality of mutation events may be involved in creating antigen binding activity. However, it is rare that a particular amino acid mutation at a particular position is absolutely required in order to create antigen binding activity. Alternatives are permitted at all other locations. • Mutation events are not antigen driven, they are antigen selected. Mutations may occur throughout the variable region. Mutations not deleterious to antigen binding are tolerated. Accordingly, many mutations in a heavily mutated sequence (particularly those outside the antigen binding site, which is generally comprised within the CDRs) are irrelevant to antigen binding, and hence to clonal selection.
[illegible]	• 11D10 light chain variable region has at least 7 point differences from the prototype VJ sequence. • 11D10 heavy chain variable region has at least 11 point differences from the prototype VDJ sequence. • 10 of the 18 point differences in the light and heavy chain variable regions occur *outside* the complementarity determining regions (CDRs). The 8other differences are broadly distributed amongst 4 of the 6 CDRs. • One of the two remaining CDRs comprises the heavy chain VDJ junction and shows evidence of an unusual VDJ combination or splicing event. • None of the 18 point differences are implicated in the regions of microhomology between 11D10 and HMFG, the antigen it mimics.

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The observations above and the calculations that follow are all based on data for antibody variable region *amino acid* sequences. (Possible "non-productive" mutations in the ambiguous positions of the corresponding DNA sequence are neither included nor relied upon.)

Probability calculation using conservative assumptions

- Assume only a small fraction of available V_H, D_H, J_H, V_L and J_L genes are suitable for generating an antibody specific for the immunizing antigen.
- Assume only a small fraction of splice options are suitable.
- Assume the expected distribution of the number of point mutations obtained under immunization conditions used is narrow, and number of mutations in 11D10 is typical.
- Assume as many as 17% of the amino acids may not be varied from the prototype heavy and light chain sequences for general immunoglobulin conformation, assembly and secretion. In addition are absolutely and invariably required for 11D10 to perform its function of raising Ab3. (This is highly conservative because: a) there is no evidence that the mutations present improve homology with HMFG; b) it ignores the possibility that point mutations elsewhere in the prototype sequence may be effective alternatives.)
- Assume the number of differences allowed at each of the other 15 point mutations is only 4 per position on average.
- Assume only kappa light chain (not lambda chain) is suitable.

[illegible]			
• Probability of obtaining exactly 11 mutations (Depends on mean and distribution of expected number of mutations under immunization conditions used)	*1 in*		5
• Possible number of acceptable point differences at each mutation point	*1 in*	$4^{(11-3)} \times 1^3 =$	6.6×10^4
• Possible locations of the 9 non-mandatory differences	*1 in*	$\frac{(118 \times 83\% - 3)!}{(118 \times 83\% - 8 - 3)!8!} = \frac{95!}{87!8!} =$	1.2×10^{11}
• V_H gene selection	*1 in*		5
• D_H gene selection	*1 in*		4
• J_H gene selection	*1 in*		2
• Splice options	*1 in*		20
Total Compound Probability	*1 in*		[illegible]

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Occurrence of a cell producing a 1mD10 V_H region (107 residues)			
• Probability of obtaining exactly 9 mutations under immunization conditions used	*1 in*		5
• Possible number of acceptable point differences at each mutation point	*1 in*	$4^7 \times 1^3 =$	1.6×10^4
• Possible locations of the 6 non-mandatory differences	*1 in*	$\frac{(107 \times 83\%)!}{(107 \times 83\% - 7)!7!} =$ $\frac{89!}{82!7!} =$	6.9×10^9
• V_H gene selection	*1 in*		5
• J_H gene selection	*1 in*		2
• Splice options	*1 in*		3
Total Compound Probability	*1 in*		1.7×10^{16}
Occurrence of a cell producing an entire 1mD10 variable region		*1 in*	[illegible]

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Probability calculation using non-c nservative assumptions

- Assume a moderate fraction of available V_H, D_H, J_H, V_L and J_L genes are suitable for generating an antibody specific for the immunizing antigen.
- Assume a moderate fraction of splice options are suitable.
- Assume the expected distribution of the number of point mutations obtained under immunization conditions used is moderate, and number of mutations in 11D10 is typical.
- Assume 3 amino acids may not be varied in heavy and light chain sequences (two C and one W) that are invariant between immunoglobulin domains. Assume 1 amino acid in the heavy chain is absolutely required for antigen binding.
- Assume the number of differences allowed elsewhere is 20 per position.

[illegible]			
• Probability of obtaining exactly 12 mutations (Depends on mean and distribution of expected number of mutations under immunization conditions used)	*1 in*		50
• Possible number of 12 point differences	*1 in*	20^{11} =	[illegible]
• Possible locations of the 12 differences	*1 in*	$\frac{(118-4)!}{(118-4-11)!11!}$ =	[illegible]
• Possible non-repetitive VDJ selection and splice combinations	*1 in*		10000
Total Compound Probability	*1 in*		[illegible]
[illegible]			
• Probability of obtaining exactly 12 mutations under immunization conditions used	*1 in*		50
• Possible number of 9 point differences	*1 in*	20^7 =	1.3×10^9
• Possible locations of the 9 differences	*1 in*	$\frac{(107-3)!}{(107-3-7)!7!}$ =	2.1×10^{10}
• Possible non-repetitive VJ selection and splice combinations	*1 in*		1000
• Choice of kappa or lambda chain	*1 in*		2
Total Compound Probability	*1 in*		2.7×10^{24}
[illegible]		*1 in*	[illegible]

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Number f mic required to reg nerate 11D10 antibody

It is difficult to predict precisely the total number of somatically mutated antibodies that arise from a single primary B cell. Patten et al. have suggested that the upper bound for the number of variants occurring during each immunization is 10^6. Multiple immunizations could theoretically increase this number. Some mutations, however, would render the subsequent lineage either unable to form a properly folded immunoglobulin, or would ablate antigen-binding activity.

A much clearer limit is set experimentally by the number of suitable antibody-producing cells obtained from the mouse at the time of harvesting. An ordinary mouse spleen has between 10^8 and 10^9 cells, the majority of which will not be specific for antigen. Only a proportion of specific cells are positively selected during screening. According to Malaya Bhattacharia-Chatterjee, less than 10% of wells were positive when fused cells from about 4 immunized mice were plated. This indicates that generally no more than a few, (certainly not more than 107) antibody-producing cells can be identified, fused and expanded from each immunized mouse according to the protocol used.

Using the *conservative* estimate *supra* for the number of possible antibody molecules capable of binding the immunizing antigen, the frequency of a second mouse harboring a spleen cell making antibody identical to 11D10 is no more than:

$$1 \text{ in } \frac{5 \times 10^{35}}{10^7} = 5 \times 10^{28} \text{ mice}$$